

PtdIns4P recognition by Vps74/GOLPH3 links PtdIns 4-kinase signaling to retrograde Golgi trafficking

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Targeting and retention of resident integral membrane proteins of the Golgi apparatus underly the function of the Golgi in glycoprotein and glycolipid processing and sorting. In yeast, steady-state Golgi localization of multiple mannosyltransferases requires recognition of their cytosolic domains by the peripheral Golgi membrane protein Vps74, an orthologue of human GOLPH3/GPP34/GMx33/MIDAS (mitochondrial DNA absence sensitive factor). We show that targeting of Vps74 and GOLPH3 to the Golgi apparatus requires ongoing synthesis of phosphatidylinositol (PtdIns) 4-phosphate

(PtdIns4P) by the Pik1 PtdIns 4-kinase and that modulation of the levels and cellular location of PtdIns4P leads to mislocalization of these proteins. Vps74 and GOLPH3 bind specifically to PtdIns4P, and a sulfate ion in a crystal structure of GOLPH3 indicates a possible phosphoinositide-binding site that is conserved in Vps74. Alterations in this site abolish phosphoinositide binding in vitro and Vps74 function in vivo. These results implicate Pik1 signaling in retention of Golgi-resident proteins via Vps74 and show that GOLPH3 family proteins are effectors of Golgi PtdIns 4-kinases.

Introduction

The individual cisternae of the Golgi apparatus contain distinct complements of lipids, carbohydrate-processing enzymes, transporters, and trafficking factors, and this organization underlies the function of the Golgi in protein and lipid processing and sorting. Golgi-localized phosphatidylinositol (PtdIns) 4-kinases are recognized to be key regulators of Golgi structure and function, although the relevant effectors and pathways are poorly characterized (D'Angelo et al., 2008; Mayinger, 2009). In human cells, there are multiple Golgi-localized PtdIns 4-kinases, but only one in yeast, which is composed of the Pik1 catalytic subunit and a myristoylated calcium-binding protein, Frq1 (Flanagan et al., 1993; Hendricks et al., 1999). Mutations in *PIK1* and *FRQ1* lead to cell death by impairing secretion at multiple points within the secretory pathway, possibly via misregulation of Arf GTPases (Garcia-Bustos et al., 1994; Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al.,

2000; Sciorra et al., 2005; Strahl et al., 2005). The known effectors of Golgi PtdIns 4-kinase include lipid transfer proteins (e.g., Osh1/2, Kes1, FAPP2, and CERT) and clathrin-coated vesicle adaptors (D'Angelo et al., 2008; Mayinger, 2009). It is unlikely that just these classes of effectors can adequately explain the breadth of PtdIns 4-kinase functions at the Golgi, and other effectors are postulated to exist.

Signaling by Golgi PtdIns 4-kinases is required for proper processing and transport of anterograde secretory cargo (D'Angelo et al., 2008; Mayinger, 2009), but it is not yet known if retrograde transport, which mediates retention of Golgi residents, is also subject to PtdIns 4-kinase regulation. Retrieval of resident Golgi proteins from older cisternae to younger cisternae is mediated by coatamer (COPI)-coated retrograde vesicles (Love et al., 1998; Lanoix et al., 1999; Todorow et al., 2000; Malsam et al., 2005). However, it has not been apparent how Golgi

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Abbreviations used in this paper: DOPC, dioleoylphosphatidylcholine; PM, plasma membrane; PtdIns, phosphatidylinositol; PtdIns(4,5)P₂, PtdIns (4,5)-bisphosphate; SPR, surface plasmon resonance; TEV, tobacco etch virus.

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glycosyltransferases are recognized by the retrograde-sorting machinery because they lack identified COPI-sorting signals. Yeast Vps74 has been shown to recognize the cytosolic portions of multiple Golgi glycosyltransferases, and this recognition is essential to maintain these enzymes in the Golgi (Schmitz et al., 2008; Tu et al., 2008). In addition, Vps74 is reported to interact with multiple subunits of the COPI coat, raising the possibility that it serves as an adapter that facilitates cargo packaging into retrograde COPI vesicles (Tu et al., 2008). The two human Vps74 orthologues, GOLPH3 (also named GPP34, GMx33, and MIDAS [mitochondrial DNA absence sensitive factor]) and GOLPH3-like, are components of the Golgi matrix (Wu et al., 2000; Snyder et al., 2006) and can partially substitute for Vps74 when expressed in yeast (Tu et al., 2008), suggesting that these proteins perform a conserved function at the Golgi. It is not understood how Vps74/GOLPH3 targeting and function at the Golgi is regulated.

We now report that recruitment of yeast Vps74 and human GOLPH3 to the yeast Golgi apparatus requires signaling by the Golgi-localized PtdIns 4-kinase Pik1 and specific recognition of PtdIns 4-phosphate (PtdIns4P) in a conserved binding pocket on the surface of Vps74 and GOLPH3. Golgi mannosyltransferases that depend on Vps74 for Golgi localization are not retained in the Golgi in mutant cells that express a form of Vps74 that does not bind PtdIns4P. These results reveal a previously unrecognized PtdIns4P-binding site in Vps74/GOLPH3 family proteins and link Pik1 signaling to retention of Golgi-resident proteins.

Results and discussion

Pik1 PtdIns 4-kinase and Sac1 lipid phosphatase determine Vps74 and GOLPH3 localization

To identify factors required to recruit Vps74 to the Golgi, we used fluorescence microscopy to screen a collection of yeast mutants for defects in Golgi localization of a GFP-Vps74 fusion protein. In wild-type cells incubated at 26 or 37°C, GFP-Vps74 is localized to Golgi compartments and the cytosol as previously reported (Schmitz et al., 2008; Tu et al., 2008). In temperature-conditional *pik1-83* and *frq1-1* mutants, GFP-Vps74 is mostly in the cytosol and nucleus after a 30-min incubation at the restrictive temperature (37°C; Fig. 1). When expressed in wild-type yeast cells, a GFP-tagged form of human GOLPH3 (GFP-GOLPH3) localizes to intracellular membranes more robustly than yeast GFP-Vps74 with lower cytosolic GFP signal and brighter Golgi puncta compared with GFP-Vps74-expressing cells (Fig. 1 [especially apparent in false-colored micrographs]). In a small proportion of cells (< 5%), a faint GFP-GOLPH3 signal is also apparent on the ER and/or the plasma membrane (PM). GFP-GOLPH3 is also localized predominantly to the cytosol in *pik1-83* and *frq1-1* cells incubated at the restrictive temperature (Fig. 1), suggesting that the basis of Golgi recruitment of Vps74 and of GOLPH3 is conserved from yeast to human.

Pik1 is the only Golgi-localized PtdIns 4-kinase in yeast, and GFP-Vps74 localization is not affected by mutations (*stt4-4* and *lsb6Δ*) in the two other yeast PtdIns 4-kinases, Stt4 and Lsb6, which localize to other organelles (unpublished data). Therefore, functional Golgi PtdIns 4-kinase is required for

targeting of Vps74 and GOLPH3 to the yeast Golgi apparatus. Recruitment of Vps74 and GOLPH3 could be mediated by a direct interaction with Pik1 and/or Frq1, for which there are orthologues encoded in the human genome, or alternatively, Golgi recruitment could require the product of Pik1-Frq1, PtdIns4P. A third mutant identified in our screen helped to distinguish between these two possibilities. In a *sac1Δ* mutant, GFP-Vps74 and GFP-GOLPH3 localize, in addition to the Golgi apparatus, to nuclear ER and cortical ER and/or PM (which cannot be definitively distinguished by light microscopy; Fig. 1 B). *SAC1* encodes an integral membrane phosphoinositide phosphatase that is localized to the ER and Golgi (Whitters et al., 1993; Guo et al., 1999; Hughes et al., 2000; Foti et al., 2001). Total cell PtdIns4P levels increase by up to 10-fold in *sac1* mutants (Guo et al., 1999; Rivas et al., 1999; Stock et al., 1999; Foti et al., 2001; Konrad et al., 2002), and a variety of GFP-tagged PtdIns4P-binding probes localize to the ER or PM as a result (Li et al., 2002; Roy and Levine, 2004; Faulhammer et al., 2007). In *sac1-24* mutant cells that express a catalytically inactive but otherwise stable form of Sac1 (Nemoto et al., 2000), GFP-Vps74 is also recruited to ER and/or PM (unpublished data), indicating that it is the loss of the phosphatase activity of Sac1 that is key to mistargeting of Vps74 and GOLPH3. Thus, correct Golgi localization of Vps74 and GOLPH3 requires synthesis of PtdIns4P in the Golgi by Pik1-Frq1 and maintenance of low PtdIns4P levels in non-Golgi membranes by the Sac1 lipid phosphatase.

Vps74 and GOLPH3 bind to PtdIns4P

The most straightforward interpretation of these results is that Vps74 and GOLPH3 directly bind PtdIns4P, and two different lipid-binding assays confirm this prediction (Fig. 2). In a dot blot assay (Kavran et al., 1998; Stevenson et al., 1998), in vitro-translated radiolabeled Vps74 and GOLPH3 bind to immobilized PtdIns4P. Weak signals are also observed for PtdIns3P, PtdIns5P, and PtdIns (4,5)-bisphosphate (PtdIns(4,5)P₂), particularly for GOLPH3 (Fig. 2 A). The specific PtdIns3P-binding control protein Snx3/Grd19 (Yu and Lemmon, 2001) binds only to PtdIns3P, and an unprogrammed in vitro translation reaction yields no specific signal (Fig. 2 A). A similar approach has shown that Vps74/GOLPH3 from *Drosophila melanogaster* also binds PtdIns4P (Dippold et al., 2009).

Surface plasmon resonance (SPR) was used to define affinities and specificities of phosphoinositide binding by yeast Vps74 and human GOLPH3. Dioleoylphosphatidylcholine (DOPC) vesicles with or without 3% (mol/mol) PtdIns3P, PtdIns4P, PtdIns5P, or PtdIns(4,5)P₂ were applied to an L1 sensor chip and equilibrium-binding curves for Vps74 and GOLPH3 to these phosphoinositides determined (Fig. 2, B and C). Vps74 binds specifically to PtdIns4P with a K_D value of 8.9 ± 0.3 μM. There is detectable binding to the other tested phosphoinositides at high concentrations of Vps74, but the estimated K_D values are more than fivefold weaker than for PtdIns4P (Table I). GOLPH3 binds more tightly to PtdIns4P than Vps74 with a K_D value of 2.6 ± 0.2 μM, which is consistent with the more robust targeting of GFP-GOLPH3 to the Golgi compared with GFP-Vps74. GOLPH3 also binds substantially to the other tested phosphoinositides,

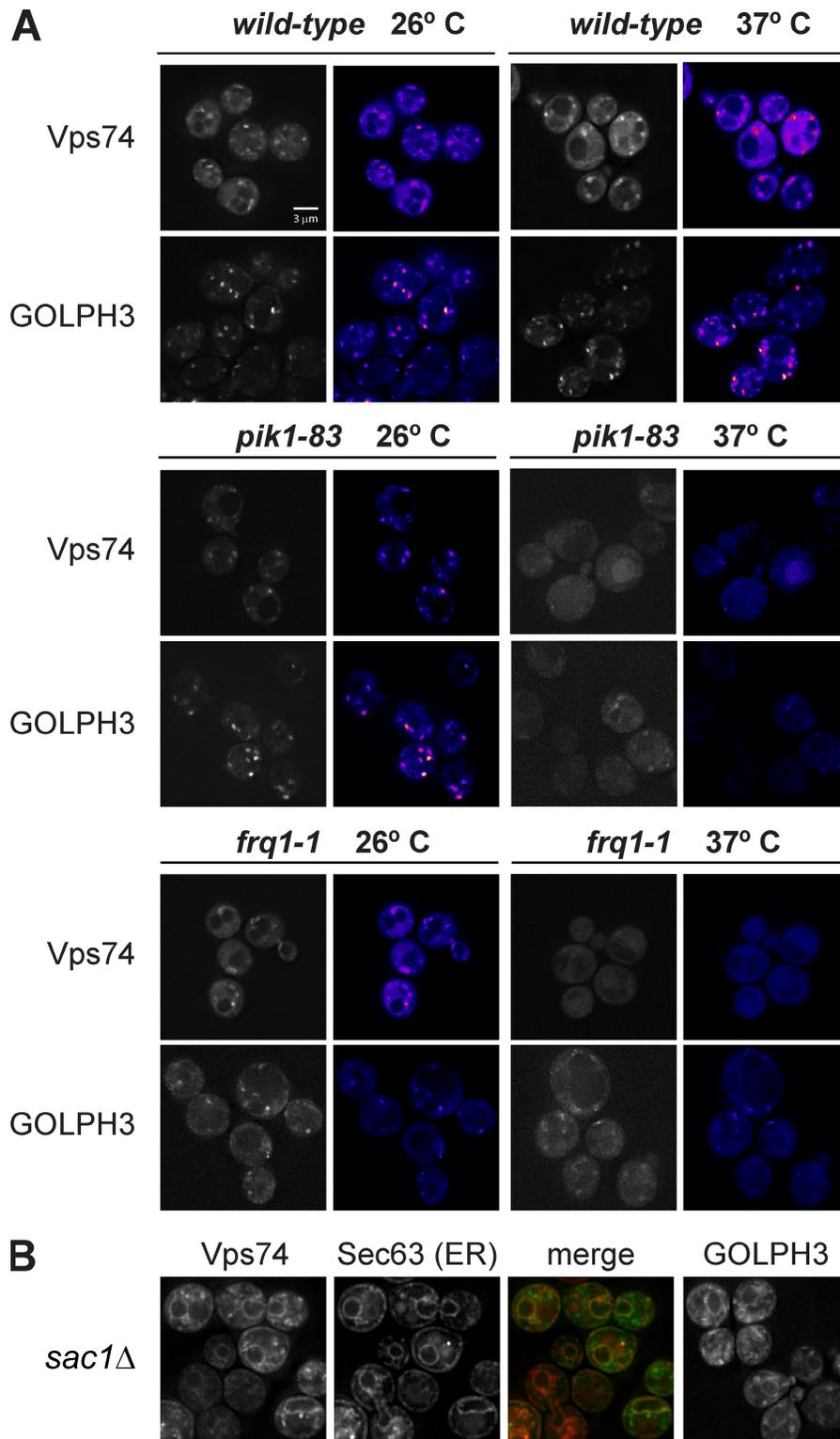


Figure 1. Intracellular targeting of yeast Vps74 and human GOLPH3 is determined by Pik1 PtdIns 4-kinase and Sac1 lipid phosphatase. (A) GFP-Vps74 or GFP-GOLPH3 was expressed in the indicated strains and visualized by fluorescence microscopy. Cultures were incubated at 26 or 37°C for 30 min before microscopy. Grayscale micrographs are not scaled equivalently so that cellular features can be better visualized. False-color images of the same micrographs are scaled equivalently so that the intensity of punctate Golgi compartments can be directly compared (from blue [lowest intensity] to yellow [highest intensity]). (B) GFP-Vps74 was expressed in *sac1Δ vps74Δ* cells that also express a resident ER protein, Sec63, fused to mCherry (Sec63-mCh). Colocalization of GFP-Vps74 and Sec63-mCherry is compared in a merged image. Localization of GFP-GOLPH3 in *sac1Δ vps74Δ* cells is shown to the right. Bar, 3 μ m.

with K_D values of $10.2 \pm 0.5 \mu\text{M}$ for binding to PtdIns3P and $\sim 25 \mu\text{M}$ for binding to PtdIns5P and PtdIns(4,5)P₂. These data confirm the cellular role of PtdIns 4-kinase in recruiting these proteins to Golgi membranes. However, it is unlikely that PtdIns4P binding alone confers specific targeting to the Golgi because other membranes also contain substantial pools of PtdIns4P (Roy and Levine, 2004).

Identification of the PtdIns4P-binding site on Vps74

We have previously reported the structure of Vps74, but the structure provided no indication of a PtdIns4P-binding site. We now report the x-ray crystal structure of human GOLPH3 to a 2.9-Å resolution. Crystals of GOLPH3 lacking the first 51 amino acids (GOLPH3 Δ 51) grew from a solution containing $\sim 0.9 \text{ M}$

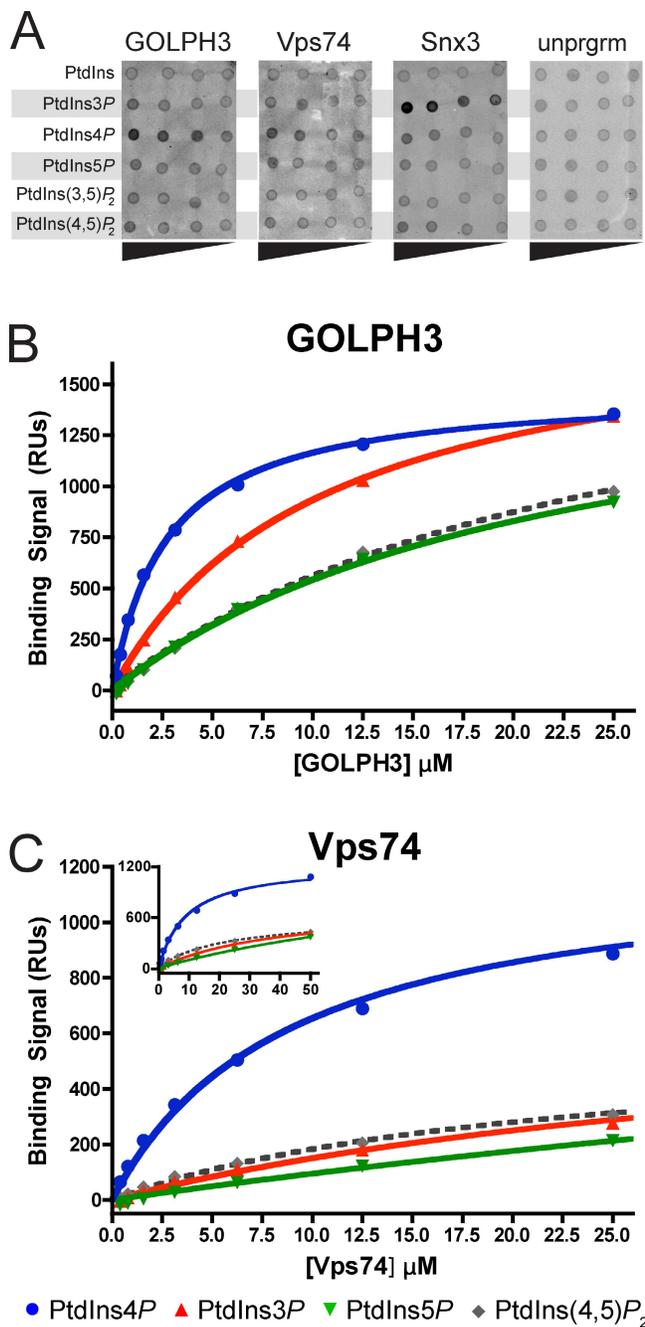


Figure 2. Specific recognition of PtdIns4P by GOLPH3 and Vps74. (A) Radiolabeled *in vitro*-translated Vps74 and GOLPH3 were incubated with nitrocellulose membranes spotted with the indicated phosphoinositides (4, 2, 1, and 0.5 μg). Bound proteins were visualized using a phosphorimager. Unprgrm, unprogramed. (B and C) SPR of GOLPH3 (B) and Vps74 (C) binding to phosphoinositides. A series of samples at the indicated concentrations was passed over an L1 chip that had been prepared with vesicles of the indicated phosphoinositides at 3% (mol/mol) in a DOPC background. The steady-state SPR response for a representative dataset, corrected for background binding to a DOPC surface, is plotted against protein concentration. The inset shows data for 50 μM Vps74. The curves indicate the fit to a simple one-site binding equation. K_D values from at least three independent binding experiments are listed in Table I. RUs, relative units.

ammonium sulfate and contain bound sulfate ions that suggest the location of a PtdIns4P-binding site.

As expected, the GOLPH3 structure is very similar to that of Vps74 (Fig. 3 and Fig. S1) based on sequence similarity

(backbone atom root mean square deviation of 1.0 Å). As observed in Vps74, a β hairpin ($\beta 3/\beta 4$) motif projects away from the helical core of the protein. This β hairpin mediates interactions in a crystallographic dimer of GOLPH3 that are equivalent to the β hairpin interface observed in the Vps74 tetramer (Fig. 3 B). Formation of the tetramer observed in the Vps74 crystals is prevented by an intermolecular disulfide bond (C84-C108), which stabilizes the GOLPH3 $\Delta 51$ crystals and hinders analysis of GOLPH3 oligomerization in solution. The primary differences between GOLPH3 and Vps74 cluster on the face opposite the β hairpin (Fig. S1 A). The $\alpha 7/\alpha 8$ loop and region between $\alpha 10$ and $\alpha 12$ of Vps74 are longer than in GOLPH3. These longer loops plus $\alpha 11$ (absent in GOLPH3) create a region on the surface of Vps74 that is much less positively charged than the equivalent surface of GOLPH3 (Fig. S1 B).

Bound anions have served as indicators for the presence of lipid headgroup-binding sites in several crystal structures, including the p47^{phox} PX (phox homology) domain (Karathanassis et al., 2002) and the Vps27 and Hrs FYVE domains (Misra and Hurley, 1999; Mao et al., 2000). We asked whether this may also be true for the sulfate ions modeled in our GOLPH3 structure. A particularly well-ordered sulfate ion lies partially buried in a positively charged pocket near the N-terminal end of $\alpha 6$ (Fig. 3 C). This pocket is also observed in the *Saccharomyces cerevisiae* Vps74 structure (Fig. 3 C and Fig. S1), and the amino acids lining this pocket are well conserved throughout fungal and animal lineages. In GOLPH3, this sulfate is stabilized by electrostatic interaction with R174, hydrogen bonds to backbone atoms of N172 and V173, and water-mediated interaction to helix $\alpha 6$. This pocket shares features with the binding sites of other structurally characterized phosphoinositide-binding modules (Fig. S1 C), conserved basic residues that could interact with the phosphates of PtdIns4P (R90, R171, and R174), and a conserved tryptophan (W81) that could pack against the inositol ring. The putative PtdIns4P-binding site of GOLPH3 is also near the hydrophobic pocket that accepts the tip of the partner β hairpin in the β hairpin interaction (Fig. 3 B). Deletion of this β hairpin, which is required for Vps74 oligomerization and function (Schmitz et al., 2008), abolishes binding to PtdIns4P (Fig. S2). This may suggest a link between oligomerization and PtdIns4P binding. Alternatively, the hydrophobic side chains in the β hairpin may stabilize binding by inserting into the hydrophobic milieu of the membrane, as has been suggested in binding of PX and FYVE domains to PtdIns3P-containing membranes (Bravo et al., 2001; Zhou et al., 2003).

PtdIns4P binding is required for Golgi targeting and *in vivo* function

To determine the functional significance of the amino acids lining this sulfate-binding pocket, we constructed variants with alanine substituted for two of the amino acids underlined in Fig. 3 C (Vps74, W88A/R97A and K178A/R181A; GOLPH3, W81A/R90A and R171A/R174A). These substitutions do not grossly affect the overall structure of the proteins: the variants are expressed in equivalent amounts compared with the wild-type proteins in yeast and *Escherichia coli*, circular dichroism spectra of wild-type and mutant Vps74 proteins are nearly identical,

Table 1. K_D values for the binding of Vps74 and GOLPH3 to phosphoinositides

Protein	PtdIns4P	PtdIns3P	PtdIns5P	PtdIns(4,5)P ₂
Vps74	8.9 ± 0.3 μM	>50 μM	>50 μM	>50 μM
GOLPH3	2.6 ± 0.2 μM	10.2 ± 0.5 μM	~25 μM	~25 μM

Values were determined from SPR (Biacore) analysis.

and the mutant Vps74 proteins retain the ability to recognize the cytosolic portions of multiple Golgi glycosyltransferases in vitro (unpublished data). By both lipid dot blot and SPR-based assays (Fig. S2), none of the Vps74 or GOLPH3 mutants detectably bind PtdIns4P or any other phosphoinositide tested, indicating that these mutations ablate PtdIns4P recognition. When expressed in *vps74Δ* and *sac1Δ* cells as fusions to GFP, the mutant forms of Vps74 and GOLPH3 do not detectably localize to

the Golgi or the ER (Fig. 4 A). Golgi function is also compromised by the mutations in *vps74*; glycoproteins secreted from cells expressing untagged forms of each Vps74 mutant are under glycosylated (Fig. 4 B), and a GFP-tagged form of a medial Golgi mannosyltransferase, Kre2-GFP, is not efficiently retained in the Golgi apparatus (Fig. 4 C), just as in *vps74Δ* cells (Schmitz et al., 2008; Tu et al., 2008). We conclude that the sulfate-binding pocket identified in the x-ray crystal structure of human GOLPH3

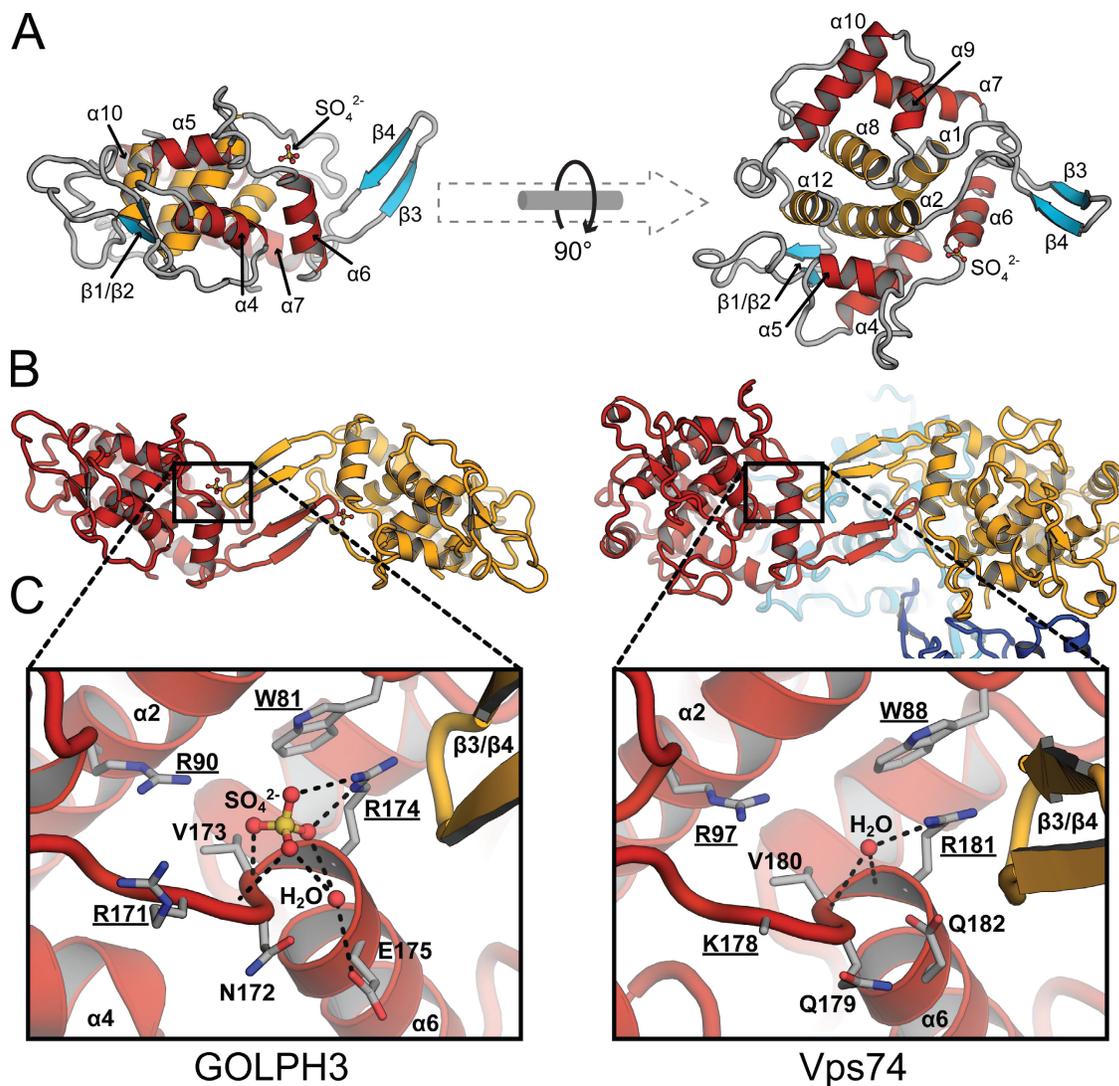
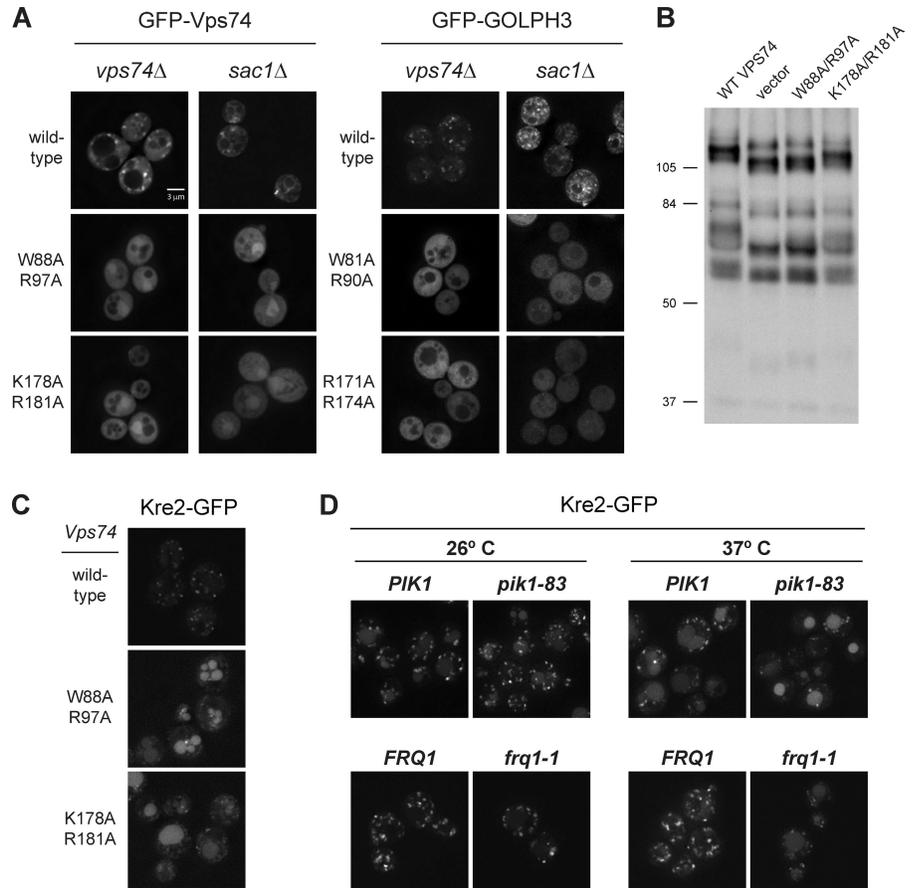


Figure 3. **Structure of human GOLPH3 and identification of a putative PtdIns4P-binding site.** (A) Orthogonal cartoons of GOLPH3. Core α helices are shown in gold (labeled in the right panel only), amphipathic α helices in red, and β strands, including the conserved $\beta 3/\beta 4$ hairpin, in blue. A well-ordered and partially buried sulfate ion is shown in stick representation. (B) The β hairpin-mediated crystallographic dimer of GOLPH3 (left) is almost identical to one of the intersubunit interfaces in the Vps74 tetramer (right). The other subunits of the Vps74 tetramer are shown in blue and cyan. (C) A magnified view of the sulfate-binding pocket (boxed regions in B) from GOLPH3, showing the sulfate and amino acids lining this pocket in stick representation. Interactions with the sulfate ion are shown with dashed lines. The same region of Vps74 is shown on the right. A water molecule occupies approximately the same position as the sulfate. The K178 side chain is disordered in the Vps74 structure. Underlined amino acids were substituted with alanine (see Results and discussion).

Figure 4. PtdIns4P binding is essential for Golgi localization of Vps74 and GOLPH3 and for Vps74-mediated retention of the Kre2 Golgi mannosyltransferase. (A) GFP-tagged forms of Vps74 were expressed in *vps74Δ* and *vps74Δ sac1Δ* cells and visualized by fluorescence microscopy. (B) SDS-PAGE autoradiography was used to compare the relative mobilities of radiolabeled proteins that are secreted into the medium by cells that express wild-type VPS74, an empty vector, or the indicated Vps74 point mutants. The migration of mass standards (kD) is indicated. (C) Localization of Kre2-GFP, a medial Golgi mannosyltransferase that requires functional Vps74 to be retained in the Golgi, is shown in *vps74Δ* cells that express wild-type or the indicated untagged Vps74 point mutants. (D) Kre2-GFP localization is shown in *pik1-83* and *frq1-1* cells grown at 26°C (permissive temperature) or incubated for 60 min at 37°C (restrictive temperature). Maximum intensity projections of z stacks are shown. Each pair of micrographs (isogenic wild type and mutant) was identically scaled so that the intensity of Kre2-GFP signals can be directly compared. Bar, 3 μm.



is critical for PtdIns4P binding and Golgi targeting of GOLPH3 and Vps74 and that PtdIns4P binding is essential for the function of Vps74. Given the conservation of residues in the PtdIns4P-binding pocket through evolution and the recent demonstration using lipid-blotting assays that yeast, fly, and human GOLPH family proteins bind PtdIns4P (Dippold et al., 2009), we suggest that PtdIns4P recognition is a common feature of all eukaryotic GOLPH3 family proteins.

Signaling by yeast Pik1 in the Golgi is generally considered to promote anterograde trafficking (Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000; Schorr et al., 2001; Sciorra et al., 2005); however, our results predict that Vps74-dependent retrograde trafficking should also require Pik1. To test this, we determined Kre2-GFP localization in *pik1-83* and *frq1-1* cells before and after inactivation of PtdIns 4-kinase activity by temperature shift (Fig. 4 D). In both mutants, the intensity of Kre2-GFP Golgi puncta decreases upon incubation at the restrictive temperature, and the intensity of GFP fluorescence in the vacuole lumen increases. This result indicates that Kre2-GFP fails to be efficiently retained in the Golgi when Pik1-mediated PtdIns4P synthesis ceases, implicating Pik1 signaling in retrograde trafficking of Golgi residents.

Stt4, the PM-localized PtdIns 4-kinase in yeast, is reported to produce the PtdIns4P that accumulates in a *sac1Δ* mutant (Audhya et al., 2000), and consistent with this, several PtdIns4P-binding GFP-pleckstrin homology domain fusion proteins are recruited to the PM in *sac1Δ* cells (Yu and Lemmon, 2001; Roy

and Levine, 2004). Our results show robust ER targeting of Vps74 and GOLPH3 in *sac1Δ* cells, which has also been observed for the pleckstrin homology domain of human CERT fused to GFP (Roy and Levine, 2004), suggesting that Sac1 acts on PtdIns4P that is produced by both Stt4 and Pik1. How does Vps74 discriminate between PtdIns4P in various organelle membranes? We speculate that this specificity is conferred by coincident recognition of PtdIns4P and Golgi glycosyltransferases, which is in line with the general paradigm of lipid-binding proteins using multivalent interactions to target specific organelles (Lemmon, 2008). Consistent with this, we observe that multiple Golgi residents that are recognized by Vps74 accumulate in the ER in *sac1Δ* cells (unpublished data), possibly because of compromised folding capacity in the ER (Mayinger et al., 1995; Kochendörfer et al., 1999; Jonikas et al., 2009). Thus, two of the known binding partners for Vps74, PtdIns4P and glycosyltransferases, are present at elevated levels in the ER in *sac1Δ* cells. A rigorous test of coincidence detection of Golgi residents and PtdIns4P by Vps74 will require delineation of the binding sites for each ligand, and the results presented in this study are a key step toward this goal.

Given the similar structures and common cellular attributes of yeast Vps74 and human GOLPH3, it is likely that these proteins execute similar functions in yeast and human cells. Indeed, human GOLPH3 and GOLPH3-like proteins can partially substitute for yeast Vps74 when expressed in a yeast mutant lacking endogenous Vps74 (Tu et al., 2008; unpublished data).

Human GOLPH3 was recently reported to associate with a non-conventional myosin, MYO18A, to facilitate flattening of TGN cisternae and budding of tubules from the TGN (Dippold et al., 2009). The yeast genome does not encode an MYO18A orthologue, but a nonconventional myosin, Myo2, does function in secretory vesicle biogenesis at the TGN (Lipatova et al., 2008), and it will be of interest to determine whether Vps74 plays a role in this process. In addition, amplification of the GOLPH3 locus in a variety of human tumors is implicated in oncogenic transformation via changes in mTOR1 signaling (Scott et al., 2009). A link between cellular transformation and the functions of Vps74 and GOLPH3 in Golgi homeostasis is not obvious. Because GOLPH3 binds PtdIns3P with just threefold reduced affinity compared with PtdIns4P, it is possible that when its expression is increased, GOLPH3 might be recruited to endosome membranes containing PtdIns3P and perturb cellular pathways that are not normally regulated by GOLPH3. It will be important to determine whether phosphoinositide recognition is crucial for GOLPH3-mediated transformation. The discovery of specific recognition of PtdIns4P by Vps74/GOLPH3 family proteins provides important insight into the roles of PtdIns 4-kinase signaling in Golgi homeostasis and may also be relevant for understanding the contribution of GOLPH3 to cancer.

Materials and methods

Yeast methods

Yeast strains were grown in standard media as required for selection of transformants and maintenance of plasmids. Deletion strains in the BY4742 (*MAT α his3-1, leu2-0, met15-0, and ura3-0*) parental background were purchased from Thermo Fisher Scientific or were constructed in BY4742 (*MAT α his3-1, leu2-0, met15-0, and ura3-0*) by integration of gene-targeted, PCR-generated DNAs (Longtine et al., 1998). The CWY94 (BY4742 *sac1 Δ ::KANMX vps74 Δ ::HISMX*) strain was constructed by sporulation and tetrad dissection, and the deleted loci were confirmed by a PCR-based test. Many of the temperature-conditional strains used in the initial screen were provided by C. Boone (University of Toronto, Toronto, Ontario, Canada) and constructed in Y7092 (*MAT α can1 Δ ::STE2pr-Sp_his5 lyp1 Δ his3 Δ 1 leu2 Δ 0 ura Δ 0 met15 Δ 0*). The *pik1-83* strain was provided by D. DeWald (Utah State University, Logan, UT), and the *sac1-24* strain was provided by V. Bankaitis (University of North Carolina, Chapel Hill, NC).

The screen for mutants that affect Vps74 localization was conducted by transforming mutant strains with a centromeric plasmid encoding N-terminal GFP-tagged Vps74 (Schmitz et al., 2008) and visualizing GFP-Vps74 by fluorescence microscopy after growth in liquid medium to an OD₆₀₀ of ~0.6. For temperature-conditional mutants, cultures were shifted to 37°C for 30 min before visualizing GFP-Vps74. A complete list of strains that were screened is available upon request. Whole cell secretion assays were performed as previously described (Gaynor and Emr, 1997).

Fluorescence microscopy

Cells were mounted in growth medium, and 3D image stacks were collected at 0.4- μ m z increments on a DeltaVision workstation (Applied Precision) based on an inverted microscope (IX-70; Olympus) using a 100 \times NA 1.4 oil immersion lens. Images were captured at 23°C with a 12-bit charge-coupled device camera (CoolSnap HQ; Photometrics) and deconvolved using the iterative-constrained algorithm (Agard, 1984) and the measured point spread function. Images were scaled and false colored using ImageJ (version 1.38; National Institutes of Health). One image from the approximate center of each z stack is shown in Figs. 1 and 4 unless noted otherwise.

Molecular biology methods and reagents

Standard molecular biological protocols and reagents were used. Mutagenesis of *VPS74* and *GOLPH3* was accomplished by PCR. All PCR-generated DNA was sequence verified. The monoclonal antibodies used were anti-GFP (1:2,500; Covance) and sheep anti-mouse HRP conjugate (1:3,000; GE Healthcare).

Protein expression and purification

Vps74 and GOLPH3 plus site-specific variants were expressed either from a pET-28a vector (EMD) to direct expression of N-terminally His₆/T7 tag proteins (T7-Vps74 and T7-GOLPH3) or from a modified pET-21a vector (EMD) to produce protein with an N-terminal His₆ tag followed by a tobacco etch virus (TEV) protease site (H₆-Vps74 and H₆-GOLPH3). Proteins were expressed in BL21 (DE3) *E. coli* (T7 tagged; EMD) or Rosetta2(DE3)plysS *E. coli* (H₆ tagged; EMD) by IPTG induction at 37°C. Selenomethionine-labeled H₆-GOLPH3 Δ 51 (SeMet-GOLPH3 Δ 51) lacking the N-terminal 51 amino acids of GOLPH3 was expressed in B834(DE3) *E. coli* (EMD) in simplified minimal medium (Guerrero et al., 2001). Cells were lysed by sonication in 25 mM Na₂HPO₄/NaH₂PO₄, 250 mM NaCl, and 10% glycerol, pH 8.0, and clarified lysates were applied to a nickel-nitrilotriacetic acid (QIAGEN) affinity column. TEV protease (150 μ g and 5 mg Vps74) was added followed by dialysis against 50 mM Tris, 50 mM NaCl, and 1 mM DTT, pH 8.0. TEV-digested Vps74 was purified by anion exchange (Source Q; GE Healthcare) and size exclusion chromatography (Superose 12; GE Healthcare) and concentrated to 10 mg/ml in 10 mM Hepes, 150 mM NaCl, and 1 mM TCEP (tris[2-carboxyethyl]phosphine), pH 7.5. GOLPH3 proteins were purified similarly with the substitution of 10 mM Hepes, pH 7.5, with 10 mM bicene, pH 8.5, in all buffers and were concentrated to 10 mg/ml in 10 mM bicene, 300 mM NaCl, and 5 mM TCEP, pH 8.5.

Lipid-binding assays

The protocol for the lipid dot blot assays is based on the method of Kavran et al. (1998) with minor modifications. The lipids (Echelon Corp) listed in Fig. 2 A were spotted in a titration series (4, 2, 1, and 0.5 μ g) onto nitrocellulose and dried. Blots were blocked in 3% fatty acid-free BSA in TBS (50 mM Tris HCl, pH 7.5, and 150 mM NaCl) at room temperature for 4 h. Labeled proteins were expressed from cloned genes (2 μ g pET-28a-based plasmid DNA) using coupled T7 transcription/translation in rabbit reticulocyte lysate (Promega) in the presence of 40 μ Ci [³⁵S]methionine (PerkinElmer). The relative labeling of the proteins was determined by quantifying radioactivity of full-length proteins after electrophoresis by phosphorimager. For blotting, 80–150 μ l of the translation reaction was mixed with 8 ml TBS containing 3% fatty acid-free BSA and 0.05% Tween 20 and incubated with lipid blots with gentle rocking at room temperature for 2 h. The membranes were washed five times in 30 ml TBS plus 0.05% Tween 20 and exposed to a phosphorimager screen. Blot images were scaled using ImageJ.

SPR-binding experiments were performed on a biosensor instrument (3000; Biacore) at 25°C in HBS buffer (25 mM Hepes and 150 mM NaCl, pH 7.5). Lipid vesicles containing 3% PtdIns3P, PtdIns4P, PtdIns5P, or PtdIns(4,5)P₂ (Cell Signaling Technology) in a DOPC (Sigma-Aldrich) background were prepared using cycles of freeze/thaw with sonication followed by extrusion through a 0.1- μ m membrane and were immobilized on a primed L1 chip (Biacore; Narayan and Lemmon, 2006). Binding response values are reported after reference (DOPC surface binding) subtraction. Data were analyzed using Prism (version 4; GraphPad Software, Inc.).

Crystallographic methods

SeMet-GOLPH3 Δ 51 crystals were grown by hanging drop vapor diffusion in drops of equal volumes of 10 mg/ml protein and crystallization buffer of 0.9 M (NH₄)₂SO₄ and 0.1 M MES, pH 6.0, suspended over crystallization buffer. Crystals (200 \times 200 \times 200 μ m) were transferred to crystallization buffer containing 15% ethylene glycol and flash frozen in liquid nitrogen. X-ray diffraction data were collected at the Advanced Photon Source (beamline 23-ID-B). Data were processed with the HKL-2000 system (Otwinowski and Minor, 1997). Multiwavelength anomalous diffraction data were collected, and an initial low resolution (3.6 Å) experimental electron density map was determined. The structure was solved by molecular replacement with a complete dataset collected to 2.9-Å resolution (Table S1) in the program Phaser (McCoy et al., 2007) using chain A from the structure of Vps74 (Protein Data Bank [PDB] accession no. 2ZIH) as the search model. The GOLPH3 Δ 51 model was built in Coot (Emsley and Cowtan, 2004) and refined using REFMAC (Collaborative Computational Project Number 4, 1994) and CNS (Brunger et al., 1998). Correct tracing of features of GOLPH3 Δ 51 distinct from the yeast homologue were confirmed in the low resolution experimental map. Crystallographic statistics are summarized in Table S1. Coordinates of GOLPH are available in PDB (accession no. 3KN1).

Online supplemental material

Fig. S1 shows a comparison of the GOLPH3 and Vps74 crystal structures and electrostatic charge distributions, and it also compares the sulfate-binding

pocket in GOLPH3 with the binding sites for phosphoinositides in other lipid-binding domains. Fig. S2 shows lipid dot blot and SPR-binding data for Vps74 and GOLPH3 bearing alanine substitutions in the putative PtdIns4P-binding pocket and an autoradiogram of in vitro-translated proteins that were used for lipid dot blots. Table S1 lists the crystallographic data collection and refinement statistics. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200909063/DC1>.

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